Isolation of *mtpim* Proves *Tnt1* a Useful Reverse Genetics Tool in *Medicago truncatula* and Uncovers New Aspects of *AP1*-Like Functions in Legumes[1]

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Comparative studies help shed light on how the huge diversity in plant forms found in nature has been produced. We use legume species to study developmental differences in inflorescence architecture and flower ontogeny with classical models such as *Arabidopsis thaliana* or *Antirrhinum majus*. Whereas genetic control of these processes has been analyzed mostly in pea (*Pisum sativum*), *Medicago truncatula* is emerging as a promising alternative system for these studies due to the availability of a range of genetic tools. To assess the use of the retrotransposon *Tnt1* for reverse genetics in *M. truncatula*, we screened a small *Tnt1*-mutagenized population using degenerate primers for MADS-box genes, known controllers of plant development. We describe here the characterization of *mtpim*, a new mutant caused by the insertion of *Tnt1* in a homolog to the *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*)/*APETALA1* (*AP1*)/*SQUAMOSA* genes. *mtpim* shows flower-to-inflorescence conversion and altered flowers with sepals transformed into leaves, indicating that *MtPIM* controls floral meristem identity and flower development. Although more extreme, this phenotype resembles the pea *pim* mutants, supporting the idea that *M. truncatula* could be used to complement analysis of reproductive development already initiated in pea. In fact, our study reveals aspects not shown by analysis of pea mutants: that the mutation in the *AP1* homolog interferes with the specification of floral organs from common primordia and causes conversion of sepals into leaves, in addition to true conversion of flowers into inflorescences. The isolation of *mtpim* represents a proof of concept demonstrating that *Tnt1* populations can be efficiently used in reverse genetics screenings in *M. truncatula*.

Recent studies have underlined the importance of comparative studies when trying to understand the evolution and divergence of different plant forms and shapes in nature (Vandenbussche et al., 2003a; Irish and Litt, 2005). Legumes are one of the largest plant families on earth and are second only to grasses in economic importance. One important agronomic trait is their ability to fix atmospheric nitrogen in symbiosis with bacteria of the genera Rhizobium, Sinorhizobium, and Azorhizobium (Schultze and Kondorosi, 1998). Development in many species of this family presents general and interesting characteristics that cannot be studied in other model species, such as Arabidopsis (*Arabidopsis thaliana*). Examples of such traits are leaf complexity, complex inflorescence architecture, and very different floral ontogeny and morphology (Hofer et al., 1997; Ferrándiz et al., 1999; Singer et al., 1999; Benlloch et al., 2002; Tucker, 2003).

We are interested in the study of flower and inflorescence architecture in legume species. Flower development has been subjected to detailed analysis in species such as Arabidopsis or *Antirrhinum majus*, but many questions can only be answered by genetic analysis of the process in species with different floral and inflorescence architectures. The inflorescence of legume species, such as garden pea (*Pisum sativum*) or *M. truncatula*, is more complex than that seen in Arabidopsis and Antirrhinum. Moreover, previous comparative studies revealed that the development of legume flowers also dramatically differs from these model species (Ferrándiz et al., 1999; Benlloch et al., 2002; Tucker, 2003). The existence of common primordia to petals and stamens in both legume species, in

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contrast to the independent origin of these organs in Arabidopsis and Antirrhinum, is one of the intriguing differences that these studies have revealed. These common primordia are four ephemeral meristems that appear between sepal and carpel primordia, subsequently dividing in a stereotyped pattern to produce a specific number of petal and stamen primordia. Thus, once the common primordia have formed, genetic information has to be provided to both specify the pattern of organ primordia initiation and the identity of these primordia.

These species differences suggest the existence of new regulatory genes in legumes or the modification of the function and/or regulation of the legume homologs of genes that control inflorescence and flower development in the model species. Clear examples of the acquisition of new functions by legume genes are *UNIFOLIATA* (*UNI*) and *STAMINA PISTILLOIDA* (*STP*), which are the pea homologs of *LEAFY/FLORICAULA* and *UNUSUAL FLORAL ORGANS/FIMBRIATA*, two key regulators of flower development in both Arabidopsis and Antirrhinum, respectively. Whereas these genes only regulate flower development in Arabidopsis and Antirrhinum, in pea they not only regulate flower development, but also compound leaf development (Hofer et al., 1997; Taylor et al., 2001). Another possible example would be the proposed role of the homologs of the genes that control floral organ identity in Arabidopsis in establishing the correct differentiation of organs from common primordia (Ferrándiz et al., 1999). This hypothesis was based on the observation that pea floral mutants with phenotypes that resemble those caused by mutations in A-, B-, or C-function genes in model species were affected not only in floral organ identity, but also in development of common primordia. For instance, in the C-type mutant *petalosus*, subdivision of common primordia is abnormal, leading to the production of an increased number of organs; in *stamina pistilloida*, with a B-type phenotype, common primordia are larger and some of them behave as new floral meristems; and in *callix carpellaris*, with a phenotype similar to the Arabidopsis *apetala2* mutants, common primordia were smaller or did not develop. Nevertheless, the involvement of A-, B-, or C-function genes in common primordia development needs experimental confirmation because the genes affected in most of these mutants have not been identified. Moreover, the *STP* gene turned out not to be a B-function gene, but rather a regulator of B function.

Many of the key regulators of inflorescence and flower development belong to the family of MADS-box genes; this has been widely demonstrated not only for model plants, but also for many other species (Theissen et al., 2000, 2001). MADS-box genes homologous to the key regulators of flower development in Arabidopsis have recently been identified in several model legume plants, but the function of most of these is unknown (Dong et al., 2005; Hecht et al., 2005). So far, those analyzed have been in pea, due to a long tradition of genetic studies and to the existence of mutant collections (Reid et al., 1996; Berbel et al., 2001, 2005; Taylor et al., 2002; Hecht et al., 2005). However, functional studies are difficult in pea because the availability of efficient reverse genetics tools in this species is very limited. The emerging model legume *M. truncatula* is a good choice of species to advance in these functional studies because it is genetically very close to pea (Choi et al., 2004; Wojciechowski et al., 2004) and presents a similar inflorescence architecture and floral ontogeny (Benlloch et al., 2002). This suggests that genetic regulation of inflorescence and flower development could be very similar between pea and *M. truncatula*; however, because no genes controlling inflorescence or flower development have been characterized in *M. truncatula*, this remains to be demonstrated.

In the last few years, *M. truncatula* and *Lotus japonicus* have emerged as model species for the study of legume biology because of their small diploid genomes, short generation times, self fertility, and relative ease of genetic transformation (Cook, 1999; Udvardi et al., 2005). Large tagged mutant populations are of great value for functional studies, as demonstrated by the use of large T-DNA and transposon-tagged mutant collections in model species such as Arabidopsis (Azpiroz-Leehan and Feldmann, 1997). The use of retrotransposons for large-scale mutagenesis in model plants has been described before (Okamoto and Hirochika, 2000; Hirochika, 2001; Yamazaki et al., 2001). The retrotransposon *Tnt1* from tobacco (*Nicotiana tabacum*; Grandbastien et al., 1989) has been shown to effectively transpose in Arabidopsis and *M. truncatula* (Courtial et al., 2001; d'Erfurth et al., 2003). The mechanism of transposition of *Tnt1* requires a cytoplasmic phase and this ensures its random dispersion in the genome. Further, the possibility of reactivating the *Tnt1* transposon by in vitro culture makes it feasible to generate large-scale *Tnt1*-tagged mutant populations of *M. truncatula* (www.eugrainlegumes.org) and these have been proposed as useful tools for reverse genetics screening in this species (d'Erfurth et al., 2003; Tadege et al., 2005). However, to our knowledge, to date no mutants or genes have been isolated and characterized based on *Tnt1* mutagenesis in Arabidopsis, *M. truncatula*, or tobacco itself, and the isolation of mutants through reverse genetics has not been described in *M. truncatula.*

RESULTS

Reverse Genetics Screening of a *Tnt1* Insertion Population *of M. truncatula*

To isolate *Tnt1* insertions in MADS-box genes, we screened a population of 200 *M. truncatula* plants containing random insertions of the *Tnt1* retrotransposon. These plants had been generated by transforming the *M. truncatula* R108 line (d'Erfurth et al., 2003) with the

tnk23 *Tnt1* construct described by Lucas et al. (1995). Previous analysis of this population had shown that the genome of each transformed plant contained between 15 and 20 *Tnt1* copies, on average (d'Erfurth et al., 2003). To test whether tagged genes could be found by a PCR-based reverse genetics approach in this population, we designed degenerate primers based on the sequence of *M. truncatula* MADS-box genes present in public databases. We designed three forward and two reverse degenerated primers for the MADS-box conserved region and used them for PCR reactions in combination with primers from both sides of the long-terminal repeats (LTR) of the *Tnt1* retrotransposon. The PCR reactions were done using genomic DNA from the 200 independent *Tnt1*-carrying transgenic plants mixed in 20 pools, each containing DNA from 10 plants. In this experiment, a PCR product of 227 bp was obtained in one DNA pool using the combination of MAD2 and LTR6 primers (Fig. 1). The same PCR reaction performed on individual plants from this pool identified plant line tnk148 as a candidate for a *Tnt1* insertion in a MADS-box gene. A part of the 227-bp PCR product corresponded to a small fragment of 128 bp identical to the beginning of the coding sequence of the pea MADS-box gene *PROLIFERATING INFLORESCENCE MERISTEM* (*PIM*; or *PEA MADS-BOX 4*), the likely ortholog of the *APETALA1* (*AP1*) gene in Arabidopsis (Mandel et al., 1992; Berbel et al., 2001; Taylor et al., 2002). The rest of the amplified sequence (99 bp) corresponded to the border of the LTRs of *Tnt1* and confirmed that the amplified fragment represented an insertion of this retrotransposon into a MADS-box gene (Fig. 1B).

To check whether the *Tnt1* insertion caused any alteration affecting plant development, T2 plants from the tnk148 line were grown in the greenhouse and their phenotype was analyzed. Around one-fourth of this population of tnk148 T2 plants exhibited a mutant phenotype that affected inflorescence architecture and flower development. In the mutant plants, flowers were replaced by a proliferation of meristems that eventually gave rise to abnormal flowers (see below).

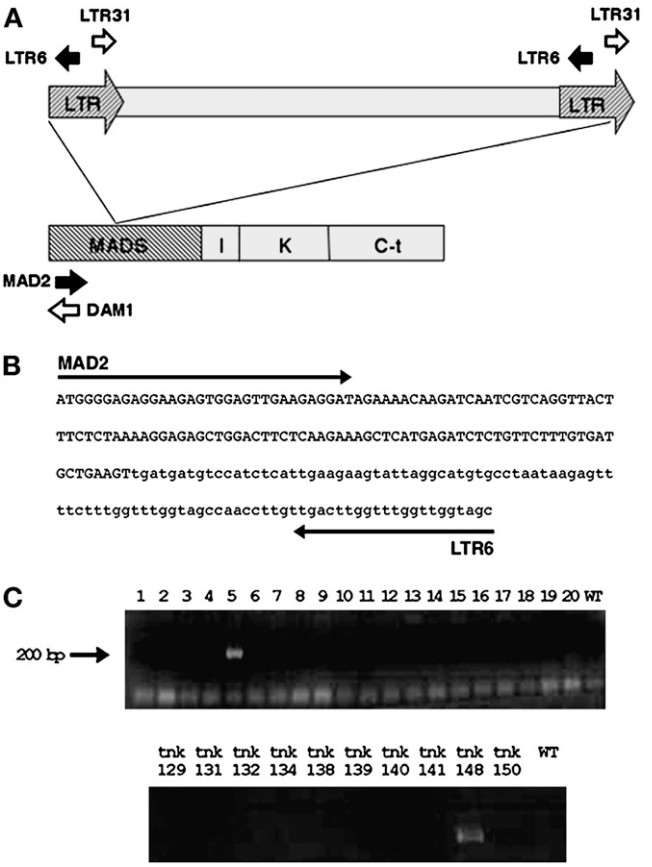

Figure 1. Identification of a *Tnt1* insertion in a MADS-box gene. A, Schematic diagram of the *Tnt1* element (5.3 kb) with flanking LTRs and a consensus MADS-box gene (approximately 0.7 kb), where conserved regions are shown (MADS-box, intermediary region, K domain, and C-terminal domain). Position and orientation of primers used for screening the population are shown. B, Sequence of the PCR product amplified in pool number 5 with primers LTR6 and MAD2. Sequence in capital letters corresponds to the *M. truncatula* genome sequence, whereas the lowercase sequence corresponds to the LTR sequence in *Tnt1* element. C, PCR amplification products with primers LTR6 and MAD2 in the *Tnt1* population DNA pools 1 to 20 (top); and in DNA extracted from individual plants from pool 5 (bottom).

Molecular Characterization of the *Tnt1* Insertion

Sequence similarity and the phenotype observed in the progeny suggested that the *Tnt1* element was inserted in a gene homologous to *PIM/AP1*. To better characterize the *Tnt1* insertion detected in the tnk148 line, we used a fragment of the *PIM* gene corresponding to the C-terminal region of the polypeptide as a probe to screen a cDNA library from flowers and inflorescences of *M. truncatula*. Four cDNA clones corresponding to a gene that we named *MtPIM* were isolated. The longest cDNA clone was 1,161 bp long and contained a 720-bp-long open reading frame encoding for a 240-amino acid protein (accession no. DQ139345).

The protein encoded by the *MtPIM* cDNA (Fig. 2A) showed high similarity to PIM (95% amino acid identity), SQUAMOSA (SQUA; Huijser et al., 1992; 71% identity), and AP1 (69%). It also showed similarity to CAULIFLOWER (CAL; Kempin et al., 1995), which also belongs to the AP1/SQUA subfamily (Fig. 2B; 63%). Interestingly, as already described for the PIM protein, the polypeptide encoded by *MtPIM* does not contain the CaaX prenylation motif present in the C terminus of other AP1-like polypeptides (Yalovsky et al., 2000; Berbel et al., 2001; Fig. 2A).

In addition, we identified one bacterial artificial chromosome clone (AC144726; *M. truncatula* clone mth2-7k13) in the *M. truncatula* genomic sequence, available in the public database, containing the complete sequence of the *MtPIM* gene. The genomic sequence indicated that the *MtPIM* gene is organized in eight exons and seven introns. The comparison of

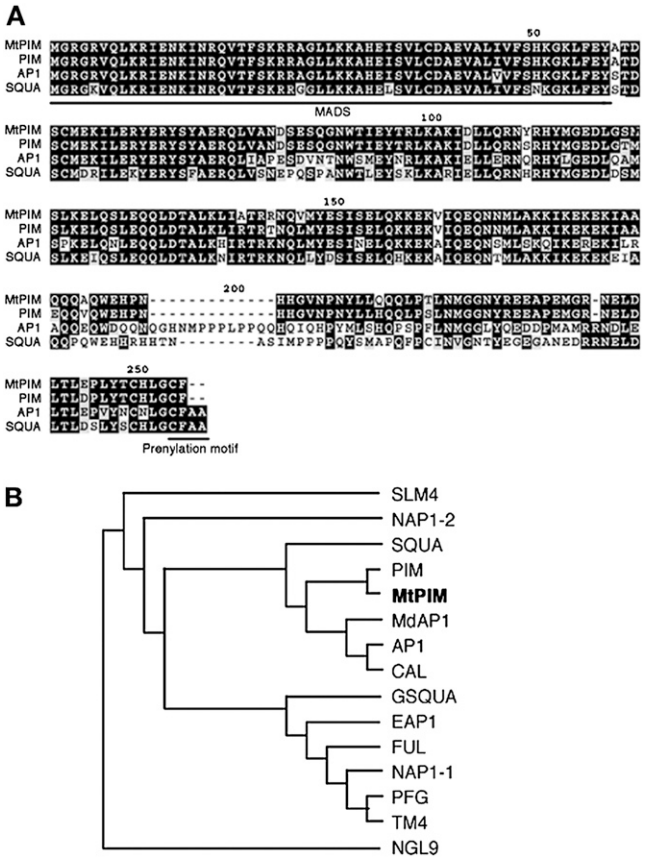

Figure 2. Sequence analysis of *MtPIM*. A, ClustalW alignment of the predicted amino acid sequences of *MtPIM* (accession no. DQ139345), *PIM*, *AP1*, and *SQUA* cDNAs. The MADS domain conserved region is underlined. The prenylation motif (CaaX) is underlined at the carboxy-terminal end of AP1 and SQUA. B, Neighbor-joining tree of the predicted amino acid sequences of genes from the *AP1*/*SQUA* subfamily. Arabidopsis: AP1 (CAA64789), CAL (AAA64789), and FUL (AAA97403); Antirrhinum: SQUA (CAA45228); Eucalyptus: EAP1 (AAG24909); Gerbera: GSQUA (CAA08805); Lycopersicon: TM4 (Q40170); Malus: MdAP1 (AAL61543); Nicotiana: NAP1-1 (AAD01422) and NAP1-2 (AAD01422); Petunia: PFG (AAF19721); Pisum: PIM (AJ279089); Silene: SLM4 (CAA56658); Medicago: MtPIM. The tree was rooted with NGL9, a *M. sativa* MADS protein with homology to members of the PISTILLATA subfamily (AF335473; Zucchero et al., 2001).

this sequence with that of the PCR product amplified from line tnk148 indicated that *Tnt1* was inserted 128 bp downstream of the ATG start codon (Fig. 3A), at the end of the first exon, in the MADS-box region. In addition, it indicated that the insertion of the *Tnt1* element created the expected 5-bp (GAAGT) duplication at the site of insertion (d'Erfurth et al., 2003).

To further confirm the *Tnt1* insertion in the *MtPIM* locus, Southern-blot analysis was performed with genomic DNA from wild-type plants and tnk148 plants exhibiting the mutant phenotype, using the complete *MtPIM* cDNA as a probe. According to the sequence of the bacterial artificial chromosome clone, in a wild-type background, a *Hin*dIII digestion probed with the entire *MtPIM* cDNA should generate two bands of 3.3 and 3.8 kb, respectively, whereas in mutant plants, the band of 3.3 kb should be shifted to 1.9 kb due to the *Tnt1* insertion (Fig. 3A). The results of this Southern-blot experiment confirmed that, in the mutant plants of line tnk148, the *Tnt1* transposable element was inserted in the *MtPIM* gene (Fig. 3B).

Cosegregation Test

To test whether the mutant phenotype cosegregated with the *Tnt1* insertion in the *MtPIM* gene, tnk148 plants with the mutant phenotype were backcrossed with the wild-type R108 line and 111 F2 plants originating from three independent backcrosses were analyzed. About one-fourth of these F2 plants (28/111 plants) exhibited the floral phenotype previously observed in T2 plants. No other phenotype associated with this group of floral mutant plants could be observed.

A sample of 83 plants from this population, including the 28 floral mutant plants, was tested by PCR for the presence or absence of the *Tnt1* insertion in the *MtPIM* locus. Plants with a wild-type phenotype either did not contain the *Tnt1* insertion in the *MtPIM* locus (19 plants) or were heterozygous for the insertion (36 plants), whereas all plants exhibiting the mutant phenotype were homozygous for the *Tnt1* insertion (data not shown). These data, along with the results of the Southern-blot analysis, strongly support the idea that the mutant floral phenotype in

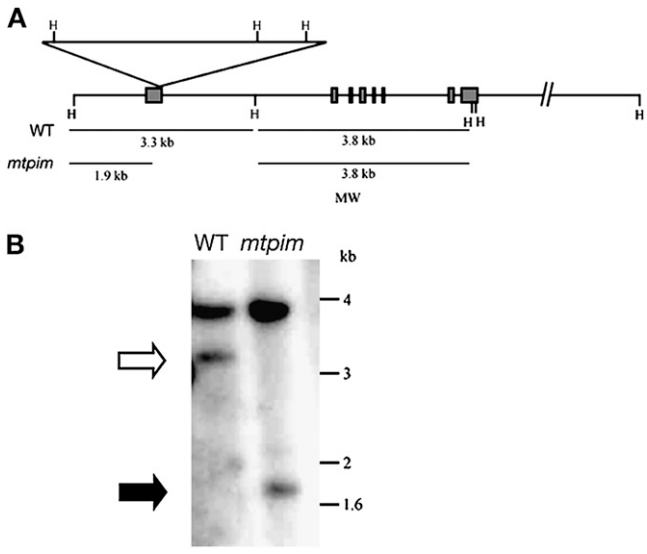

Figure 3. Structure of the *Tnt1* insertion in the *MtPIM* locus. A, Schematic diagram of the *MtPIM* gene; position of exons (boxes) and introns (lines) is shown. The *Tnt1* insertion lies at the end of the first exon. Restriction sites for *Hin*dIII are indicated (H). B, Southern blot of *Hin*dIII-digested wild-type and mutant genomic DNA hybridized with the complete cDNA of *MtPIM*. In the wild-type sample, digestion generates two hybridizing fragments of 3.3 (white arrow) and 3.8 kb. The *Tnt1* insertion in *mtpim* generates a new fragment of 1.9 kb (black arrow).

the *M. truncatula* tnk148 line was caused by the insertion of the *Tnt1* retrotransposon in the *MtPIM* gene.

Expression Pattern of the *MtPIM* Gene

Northern-blot analysis performed on RNA from different plant organs showed that *MtPIM* was specifically expressed in floral tissues, although a faint signal was also detected in stems (Fig. 4A). No expression of *MtPIM* was detected in root or leaf tissue. Expression of *MtPIM* was not detected in floral apices of the *mtpim* mutant (Fig. 4B), indicating that the mutation caused by *Tnt1* likely represents a null allele of the gene.

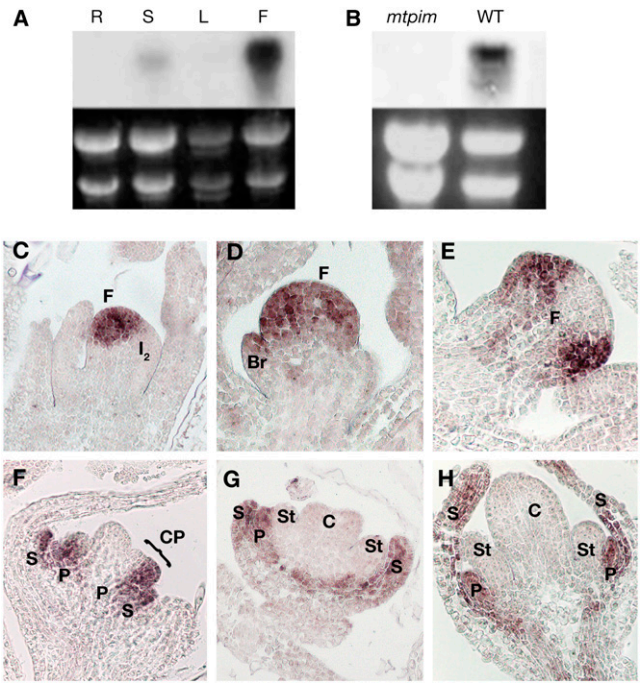

Figure 4. Expression pattern of *MtPIM*. A, Northern-blot analysis of *MtPIM* expression in roots (R), stems (S), leaves (L), and flowers (F) of wild-type *M. truncatula* plants. B, Northern-blot analysis of *MtPIM* expression in inflorescence apexes of wild-type and *mtpim* mutants. Blots in A and B were loaded with samples of 15-μg RNA. A picture of each gel stained with ethidium bromide is shown below. C to H, In situ hybridization of *MtPIM* RNA in wild-type *M. truncatula* inflorescences and flowers. C, Longitudinal section of a secondary inflorescence meristem differentiating a floral meristem. *MtPIM* is strongly expressed in the floral meristem. D, Early stage 2 floral meristem, showing uniform expression of *MtPIM*. *MtPIM* RNA can also be detected in the developing bract. E, Floral meristem at late stage 2. *MtPIM* expression is restricted to the periphery of the meristem. At this stage, no floral organ primordia have been initiated. F, Stage 4 floral meristem, where the common primordia can be observed. Expression is restricted to the region of the common primordia, which will give rise to petals. G, Floral meristem at stage 5, when petals and stamens start differentiating from the common primordia. H, In later stages (stage 6), expression is maintained in sepals and petals. F, Floral meristem; I_2, inflorescence meristem; Br, bract; CP, common primordia; P, petal; S, sepal; St, stamen; C, carpel. Developmental stages were defined according to Benlloch et al. (2002).

To detail the *MtPIM* expression pattern, in situ hybridization on wild-type inflorescence apices was performed (Fig. 4, C–H). During flower development, expression of *MtPIM* was first detected when the secondary inflorescence meristems started producing floral meristems (Fig. 4C). At stage 1 of floral meristem development (developmental stages as defined by Benlloch et al., 2002), the expression was uniform in the entire floral meristem. At this stage, expression of *MtPIM* could also be observed in the subtending bract (Fig. 4D). As the floral meristem developed, expression was soon restricted to the peripheral region of the meristem from which whorls 1 and 2 would form (Fig. 4E). At stage 4, when common primordia are first observed, *MtPIM* was expressed in sepal and common primordia. Interestingly, from the beginning, expression in common primordia was restricted to the region that would give rise to the petals (Fig. 4F). The expression of *MtPIM* was maintained through development of the flower in sepals and petals (Fig. 4G), although the signal became fainter in flowers at later developmental stages (Fig. 4H).

mtpim Shows Proliferation of Meristems, Loss of Sepal Identity, and Defects in Common Primordia Development

The insertion of Tnt1 in the *MtPIM* gene causes dramatic phenotypical changes affecting inflorescence and flower architecture (Figs. 5 and 6). The mutation seems to affect only reproductive stages of development because no effect on vegetative organ development could be observed in mutant plants.

In wild-type *M. truncatula* plants, flowers are produced by secondary inflorescence meristems (I_2) formed in the axils of the leaves produced by the primary inflorescence (I_1; Fig. 5, A and B). Each I_2 laterally produces one to three floral meristems on its flanks (usually one in *M. truncatula* R108), each subtended by a bract, and then the I_2 meristem differentiates into a residual stub or spike (Fig. 5, A and C). The architecture of the *mtpim* mutant inflorescence was dramatically altered (Fig. 5, E–G). Each floral position, subtended by the corresponding bract, was occupied by a highly branched structure. The degree of ramification of these structures increased acropetally along the main stem, the apical structures appearing in upper nodes being more branched than the basal ones.

Scanning electron microscopy (SEM) analysis of the inflorescences of the *mtpim* mutant revealed that I_2 meristems were normally produced by I_1. As in the wild type, each I_2 laterally differentiated new meristems, subtended by bracts, and then terminated as a spike (Fig. 6, C and D). However, these lateral meristems, which in the wild type gave rise to floral meristems, behaved as I_2 meristems in the mutant: Rather than developing as flowers, they again laterally produced two new meristems subtended by bracts and differentiated into a spike. The two new meristems reiterated this symmetrical division pattern,

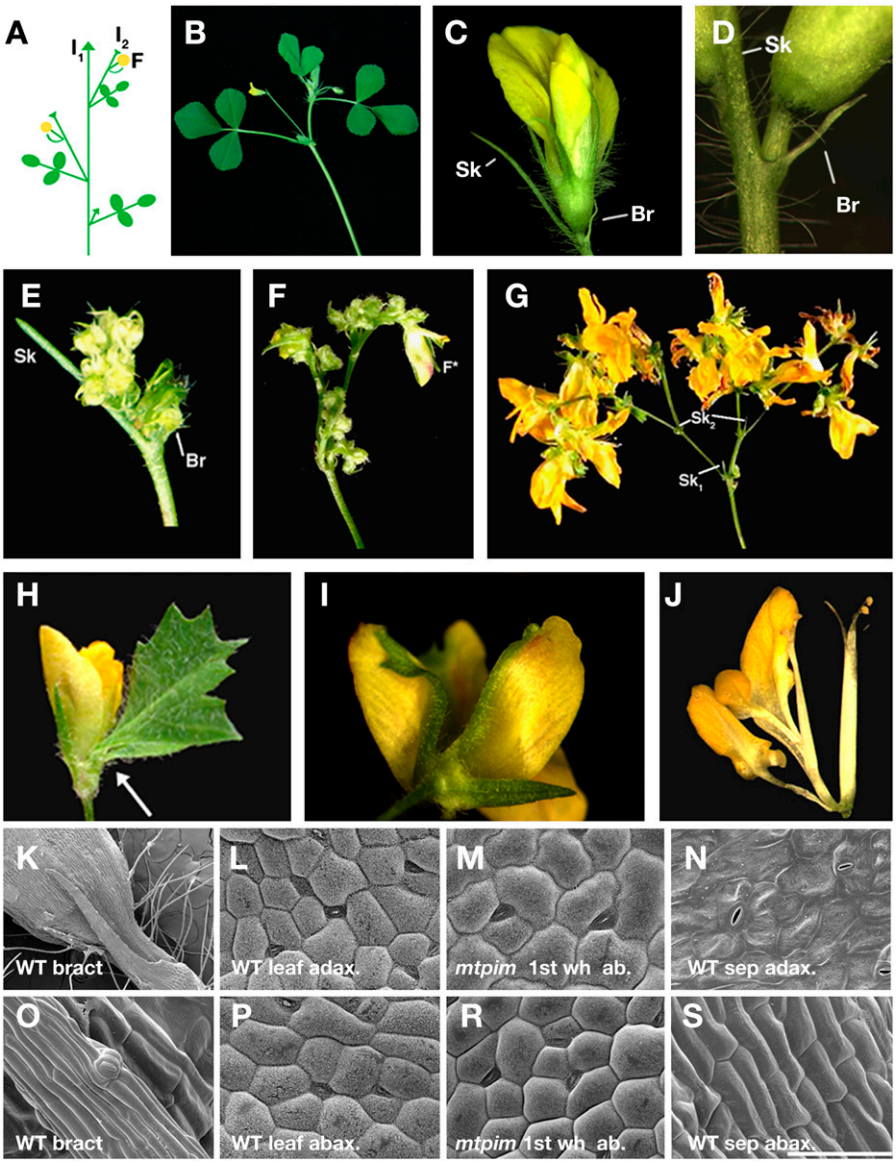

Figure 5. Floral phenotype of the *mtpim* mutant. A, Cartoon of a wild-type *M. truncatula* inflorescence. After floral transition, the shoot apical meristem becomes a primary inflorescence meristem (I_1). I_1 gives rise to secondary inflorescence meristems (I_2) in the axils of leaves, which in turn laterally differentiate flowers (F) subtended by bracts before terminating in a residual stub or spike. B, Apical part of the main stem of a *M. truncatula* plant that has gone through floral transition. The shoot apex and two secondary inflorescences, each of them subtended by a trifoliate leaf, can be distinguished. C, Close-up of a wild-type secondary inflorescence. Br, Bract; Sk, spike. D, Detail of a flower where the subtending bract is clearly visible. E, Close-up of a *mtpim* secondary inflorescence. The secondary inflorescence meristem produces a bract that subtends a proliferating structure and finally differentiates as a spike. F, Secondary inflorescence of a *mtpim* plant. After producing several proliferating meristems, an abnormal flower (F*) has finally differentiated. G, A different *mtpim* secondary inflorescence, found in a more apical position, in which the pattern of symmetrical divisions is clearly observed. After a number of divisions, most of the meristems have differentiated as flowers. H to J, Homeotic transformations in floral organs of the *mtpim* mutant. H, *mtpim* flower with transformation of a sepal into a leaf-like organ. I, Mosaic organ of sepal and petal tissues. J, Some of the stamens in the staminal tube develop petaloid extensions. K to S, SEM analysis of epidermal cell types of first-whorl leaf-like organs of *mtpim* flowers. K, View of a wild-type bract. L, Epidermis of the adaxial side of a wild-type leaf. M, Adaxial side of a leaf-like first-whorl organ of a *mtpim* flower. N, Adaxial side of a wild-type sepal. O, Close-up of a wild-type bract, showing the epidermal cell types. P, Abaxial side of a wild-type leaf. R, Abaxial side of a transformed first-whorl *mtpim* floral organ. S, Abaxial side of a wild-type sepal. Scale bar = 600 μm (K), 100 μm (O), 60 μm (L–N and P–S).

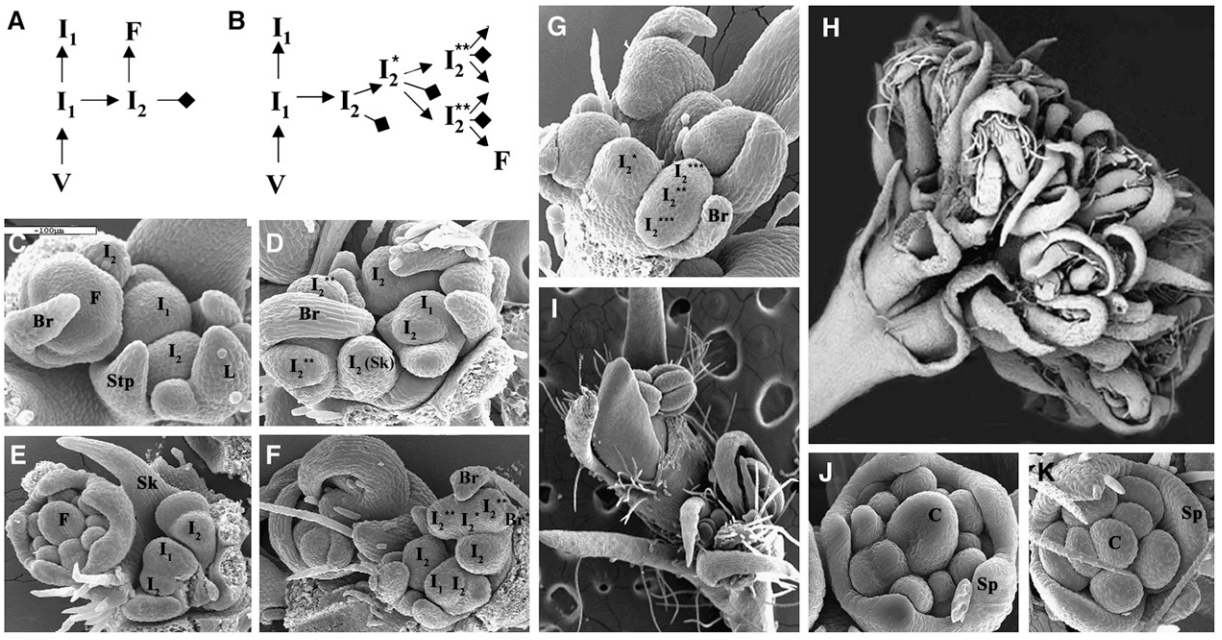

Figure 6. Development of inflorescence meristems in the wild-type and *mtpim* mutant. A and B, Schematic representation of meristem fates in *M. truncatula* wild-type (A) and *mtpim* (B) mutants. V, Vegetative shoot apical meristem; I_1, primary inflorescence meristem; I_2, secondary inflorescence meristem; F, floral meristem; ♦, spike. C and E, SEM micrographs of wild-type inflorescence apexes. C, The primary inflorescence meristem (I_1, central) is differentiating a leaf (L), with the corresponding flanking stipules (Stp), and an axillary secondary inflorescence meristem (I_2). At the top left corner, a developing secondary inflorescence shows a flower (F) subtended by a bract (Br), produced by the I_2 meristem. E, Similar structures to those shown in C are indicated. The secondary inflorescence in the top left corner shows a flower (F) in a later stage of development, where floral organ primordia are clearly visible. The I_2 has already differentiated the terminal spike (Sk). D and F, SEM micrographs of *mtpim* inflorescence apexes. D, The primary inflorescence meristem (I_1), at the right side, is differentiating a leaf and a secondary inflorescence meristem (I_2). The secondary inflorescence at the left shows noticeable phenotypic alterations. Whereas I_2 is already differentiating as a spike (Sk), in the axil of the bract (Br), instead of floral meristems, a second-order I_2 meristem (below the bract) has produced two new lateral structures, which resemble third-order I_2 meristems (I_2**). F, View of a different *mtpim* inflorescence apex. In the secondary inflorescence developing at the right side end, second- and third-order I_2 are easily distinguished (I_2*, I_2**). In the top left corner, a highly branched structure has formed. G, Close-up view of the structure formed in place of a floral meristem in a *mtpim* mutant, showing the symmetrical pattern of meristem division. H, Highly branched structure formed in a secondary inflorescence of a *mtpim* mutant. An extreme proliferation of bracts with associated meristems is observed. I, *mtpim* secondary inflorescence in which an aberrant flower has developed (top left). J and K, Flowers of wild type (J) and the *mtpim* mutant (K). J, In wild type, common primordia have already formed and divided between the sepals (Sp) and the carpel (C) to differentiate five petal and 10 stamen primordia. K, In this *mtpim* flower, common primordia have divided abnormally to give rise to a total number of seven floral organ primordia.

giving rise to the proliferating structures observed in the mutant (Fig. 6, D and F–H).

Eventually, aberrant flowers were able to differentiate from these proliferating meristems (Fig. 6I). The frequency with which this occurred increased acropetally (Fig. 5G). The number and nature of the organs in *mtpim* flowers were highly variable (Table I). Sepals were typically reduced in number and transformed into organs similar to leaves both in shape and epidermal cell types (Fig. 5, H and K–S). Second-whorl organs were reduced in number and/or presented an altered morphology. The adaxial petal was frequently the only petal formed and, occasionally, mosaic organs of sepal and petal tissues were found (Fig. 5I). Third- and fourth-whorl organs were frequently absent or reduced in number. Stamens often had patches of petaloid tissue (petaloid stamens; Fig. 5J).

SEM analysis of the development of wild-type and mutant floral meristems showed that, in the mutant, the number of floral organ primordia initiated was reduced and their production did not follow the highly structured pattern observed in wild-type floral meristems (Fig. 6, J and K). This observation agrees with the variable number and nature of floral organs observed in the second and third whorls of the mutant flowers. Whereas the *mtpim* mutation affected organ number in all floral whorls, phenotypic alterations were particularly evident in the patterning of the common primordia. In wild-type flowers, four common primordia arise between the sepal and carpel whorls and subsequently divide in a fixed and precise pattern to give rise to five petal and 10 stamen primordia (Benlloch et al., 2002). In *mtpim* mutant flowers, common primordia were formed, but did not follow the

Table I. *Phenotype of mtpim flowers: floral organs in the flowers formed in nodes 1 to 10 of wild-type and five different mtpim plants*

Floral Organs	Wild Type	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5
Flowers[a]/node	1.0 ± 0.0	1.6 ± 0.6	5.1 ± 1.6	2.4 ± 1.0	2.0 ± 0.5	4.5 ± 1.8
Proliferating structures[b]/node	0.0 ± 0.0	1.0 ± 1.5	2.6 ± 1.0	1.5 ± 0.9	1.4 ± 0.8	3.2 ± 1.2
Sepals/flower	5.0 ± 0.0	2.0 ± 0.9	0.6 ± 0.2	1.6 ± 0.8	1.4 ± 0.5	0.9 ± 0.3
Leaf-like sepals/flower	0.0 ± 0.0	0.6 ± 0.2	1.4 ± 0.4	0.5 ± 0.1	0.7 ± 0.3	1.5 ± 0.4
Sepal-petal mosaics/flower	0.0 ± 0.0	0.06 ± 0.20	0.8 ± 0.2	0.1 ± 0.4	0.3 ± 0.5	0.6 ± 0.2
Petals/flower	5.0 ± 0.0	1.3 ± 0.3	1.9 ± 0.3	1.2 ± 0.5	1.6 ± 0.2	2.3 ± 0.3
Stamens/flower	10.0 ± 0.0	3.8 ± 1.8	3.7 ± 1.3	3.0 ± 0.9	3.0 ± 1.2	3.5 ± 1.0
Petaloid stamens/flower	0.0 ± 0.0	0.1 ± 0.3	1.0 ± 0.5	0.2 ± 0.5	0.2 ± 0.5	1.0 ± 0.4
Carpels/flower	1.0 ± 0.0	0.3 ± 0.5	0.2 ± 0.3	0.4 ± 0.5	0.4 ± 0.5	0.3 ± 0.4

[a]The number of flowers and the number of different organs in these flowers were scored in the 10 first nodes of the main stem of a wild-type and five *mtpim* plants (plants 1–5). Each wild-type node normally contained an inflorescence bearing one flower, whereas the inflorescences in *mtpim* plants produced a combination of proliferating meristems and abnormal flowers. The values are mean number of each type of organ (±SE) per plant, referred to the number of flowers scored in each plant. [b]Proliferating structures, formed by a cluster of proliferating inflorescence meristems, as shown in Figure 5E.

typical pattern of divisions, producing floral organs that were reduced in number and developed in abnormal positions.

DISCUSSION

We have screened a *Tnt1*-tagged population of *M. truncatula* for insertions in MADS-box genes with the double aim of (1) evaluating the use of this kind of population as an efficient reverse genetics tool in this species; and (2) identifying genes involved in the control of *M. truncatula* reproductive development.

We describe here the isolation and characterization of the *mtpim* mutant. Our results represent a proof of concept supporting the previous proposal that *Tnt1*-tagged populations could work efficiently for reverse genetics in *M. truncatula.* We show in this work that the mutation is caused by an insertion of *Tnt1* in the *MtPIM* gene, a homolog of *AP1/SQUA/PIM*. Our analyses indicate that this gene is a key controller in *M. truncatula* of both floral meristem identity and floral development, regulating sepal identity and the development of floral organs from common primordia.

MtPIM Is a Homolog of PIM/AP1/SQUA

Our results indicate that the floral mutant phenotype observed in the *M. truncatula* tnk148 line corresponds to an insertion in *MtPIM*. Because the tnk148 line contains several *Tnt1* insertions, the possibility of a second mutation contributing to the observed phenotype has to be taken into account. However, the analysis of a large number of *mtpim* individuals from an F2 population derived from a backcross of line tnk148 into the wild-type parental line showed that the mutant phenotypes were quite homogeneous and cosegregated perfectly with the Tnt1 insertion in *MtPIM*. This would imply that, if a second gene was mutated, it would have to be tightly linked to *MtPIM*.

Moreover, as discussed below, the phenotypes of *mtpim* and *pim* are, in essence, rather similar, except that the phenotype of the *M. truncatula* mutant seems more severe than that of the pea mutant. This would mean that the second mutation would have to be affecting a gene playing a role similar to *mtpim/pim*. Therefore, although a second mutation cannot be formally discarded, we do not consider this as a likely possibility.

MtPIM is very similar to the pea gene *PIM*, a likely ortholog of the *AP1* and *SQUA* genes from Arabidopsis and Antirrhinum, respectively. The MtPIM polypeptide lacks the C-terminal prenylation motif present in other members of the AP1 family, such as AP1, CAL, or SQUA (Yalovsky et al., 2000; Berbel et al., 2001). This also occurs in PIM and supports the idea that this kind of posttranslational modification is not required for the AP1 function in legumes.

The *MtPIM* expression pattern is similar to that described for *AP1* and other *AP1*-like genes and agrees with the dual role that these genes have been proposed to play, first in specifying floral meristem identity and later in floral organ identity (Bowman et al., 1993; Berbel et al., 2001). At early stages of floral meristem development, *MtPIM* expression was observed in the developing bract that forms at the abaxial side of the floral meristem. Neither pea nor Arabidopsis flowers are subtended by bracts, but Antirrhinum flowers are, and transient expression of *SQUA* in the bract has also been described in this species (Huijser et al., 1992). However, neither *mtpim* mutation nor *squa* affect bract formation, indicating that either they are not required for bract development or, alternatively, they are redundant in this role with other factors.

MtPIM Specifies Floral Meristem Identity: The Mutation Converts Flowers into Secondary Inflorescences

In *mtpim* plants, the flowers are replaced by complex structures with inflorescence characteristics indicating that *MtPIM* is required for specification of floral meristem identity. In this view, *mtpim* flowers are replaced by shoots and the *mtpim* phenotype is equivalent to that of *ap1*, *squa*, or *pim* mutants from Arabidopsis,

Antirrhinum, and pea, respectively (Huijser et al., 1992; Bowman et al., 1993; Taylor et al., 2002). The macroscopic phenotype of *pim* led to the suggestion that in the pea mutant the flowers could be developing as secondary inflorescences (Taylor et al., 2002). Our detailed analysis by SEM clearly shows that in *mtpim* this is actually the case: Floral meristems behave like I_2 meristems. A difference between *mtpim* and *pim* is that the proliferating phenotype is more extreme in *mtpim* than in pea mutants (Taylor et al., 2002; A. Berbel, C. Ferrandiz, and F. Madueño, unpublished data). Because at least one of the pea mutant alleles, *pim-1*, corresponds to a null mutation, this difference probably reflects a higher degree of redundancy in floral meristem specification in pea.

Whereas the Arabidopsis *ap1* mutants also exhibit flower-to-inflorescence transformations, in these mutants this transformation is not complete because flowers are actually formed. *ap1* flowers have normal stamens and carpels, but petals are absent and secondary floral meristems develop in the axils of the first-whorl organs (Bowman et al., 1993). In fact, the almost complete flower-to-inflorescence conversion in pea and *M. truncatula* mutants more strongly resembles the phenotype of the Antirrhinum *squa* mutants. The strong proliferating phenotype of *mtpim* resembles that of Arabidopsis plants homozygous for mutations in both *AP1* and *CAL*, a homolog to *AP1*, only found in Brassicaceae, which acts redundantly to specify floral meristem identity (Bowman et al., 1993; Lowman and Purugganan, 1999).

A second *AP1* homolog, *PsMADS9*, exists in the pea genome (N. Carrasquilla, A. Berbel, J.P. Beltrán, and F. Madueño, unpublished data; cited by Litt and Irish, 2003). *PsMADS9*, which is more similar to *PIM* than to any other member of the AP1 clade, does not have a clearly assigned function yet because no mutant in this gene has been identified so far. This gene would be a good candidate to account for the suggested redundancy in the *AP1* function in pea. The stronger phenotype of *mtpim* compared to *pim* could be explained if such a gene was absent from *M. truncatula* or if its contribution to the *AP1* function in *M. truncatula* was lower than in pea.

Flower Development: *MtPIM* Regulates Floral Organ Identity and Patterning of Common Primordia

MtPIM plays a major role in the specification of floral meristem identity, but *mtpim* plants are able to eventually produce flowers. Initiation of these flowers is likely due to the activity of other meristem identity genes, partially redundant with *MtPIM*, such as the homologs to the genes *LEAFY* or *FRUITFULL*, which have already been identified in the sequence of the *M. truncatula* genome (Weigel et al., 1992; Ferrándiz et al., 2000; Hecht et al., 2005). The flowers formed by the *mtpim* mutant showed defects in floral organ identity and number. These defects were quite variable and frequently affected the organs of the first and second whorls. A remarkable feature of *mtpim* flowers is that sepals were often converted into organs very similar to the leaflets that constitute the *M. truncatula* trifoliate leaf, indicating that *MtPIM* is involved in the specification of sepal identity. In this respect, the *mtpim* phenotype differs from that of *ap1* or *pim* where sepals are converted into bracts, pointing again to a higher degree of redundancy in these latter species.

Organs from the second and third whorls of the *M. truncatula* and pea flowers derive from common primordia. These are four short-lived meristems that form between sepal and carpel primordia. Each of these meristems subsequently divides in a characteristic pattern to produce the petal and stamen primordia (Ferrándiz et al., 1999; Tucker, 2003). The phenotypic characterization of pea floral mutants with defects in organ identity similar to classical *abc* mutants led to the proposal that floral organ identity genes would also control common primordia development because most of these mutants showed aberrant patterning of second- and third-whorl primordia initiation in addition to homeotic changes in organ identity (Ferrándiz et al., 1999). However, to date this hypothesis has not been confirmed because the genes responsible for these mutations either remained unidentified or were not organ identity genes.

Interestingly, the *mtpim* mutation also affects the normal development of organs arising from the common primordia (i.e. petals and stamens), which were often abnormal, reduced in number, and chimeric in nature. Sepal/petal and petal/stamen chimeras are also frequently observed in the *pim* mutant (J. Hofer, personal communication; A. Berbel, C. Ferrandiz, and F. Madueño, unpublished data). These mosaic organs seem to appear more frequently in the legume mutants than in *ap1* or *squa*, possibly reflecting the common origin of these organs in pea and Medicago. Moreover, SEM analysis of developing *mtpim* flowers indicated that common primordia divided abnormally. The onset of *MtPIM* expression in common primordia occurs at early stages of development, prior to any morphological sign of petal and stamen initiation, and very precisely marks the boundaries between the regions of the common primordia that will give rise to petals and stamens. This, together with the mutant phenotype, suggests a possible role of *MtPIM* in common primordia patterning, maybe providing positional cues for correct organ initiation.

In summary, the *mtpim* mutation affects floral organ identity, as shown by the sepal-to-leaf transformations, but also seems to affect the specification of second- and third-whorl organs from common primordia, possibly by interfering with the establishment of boundaries during common primordia division.

Isolation of *MtPIM* Demonstrates That *Tnt1* Is a Useful Genetic Tool in *M. truncatula*

For species such as *M. truncatula* or *L. japonicus* to work as useful model legumes, it is necessary that

efficient reverse genetics tools are available for them. Collections of lines derived from insertional mutagenesis are a very important resource for the development of this kind of tool. T-DNA and transposon-tagged populations have been very useful for the genetic dissection of developmental processes in different plants (Carpenter and Coen, 1990; Azpiroz-Leehan and Feldmann, 1997; Vandenbussche et al., 2003b). However, as transformation of *M. truncatula* and *L. japonicus* is rather time consuming, efforts are being made to set up efficient insertional mutagenesis based on transposable elements.

Retrotransposons present advantages in comparison with T-DNAs or other transposable elements because of their random insertion in the genome and their low frequency of rearrangements or aberrant or incomplete insertions, which can make isolation of tagged mutants difficult. In *L. japonicus*, the use of LORE1, an endogenous retrotransposon recently identified, is being explored as a tool for insertional mutagenesis (Madsen et al., 2005). The tobacco retrotransposon *Tnt1* has recently been shown to efficiently transpose in *M. truncatula* (d'Erfurth et al., 2003). *Tnt1* insertions showed no site specificity, were stably transmitted to the progeny, and could be separated by segregation. All these characteristics led to *Tnt1* being proposed as a tool for insertional mutagenesis in *M. truncatula* as it previously was for Arabidopsis (Courtial et al., 2001; d'Erfurth et al., 2003). Nevertheless, to date no genes have been cloned and characterized based on *Tnt1* in Arabidopsis, *M. truncatula*, or tobacco itself.

The isolation of *MtPIM* represents proof of the concept that shows that, in fact, *Tnt1* works efficiently for gene tagging in *M. truncatula* and that *Tnt1* populations constitute a useful resource for reverse genetics. Our analysis confirmed that the *Tnt1* insertion causing the *mtpim* mutation showed no rearrangement and that the mutant phenotype is stably inherited and shows Mendelian segregation. Whereas detection of the *mtpim* mutation in a starting population of only 200 *Tnt1*-tagged lines might look fortunate, it should be considered that they were independent lines with an average of 15 to 20 insertions per line, therefore representing up to 4,000 *Tnt1* inserts. Assuming that one-third of the inserts are in genes (d'Erfurth et al., 2003), 1,300 genes could be tagged in this population. If the genome of *M. truncatula* contains 36,000 genes (Tadege et al., 2005), one of every 30 genes could be tagged in this small population. Finally, it also has to be considered that we screened using degenerate primers against the large MADS-box gene family (more than 100 genes in Arabidopsis). Although it is clear that the small size of this population does not allow statistical analysis, the frequency that could be estimated seems to be in a range that is consistent with *Tnt1* randomly inserting in the *M. truncatula* genome.

While this work confirms *Tnt1* populations as a useful resource for reverse genetics in *M. truncatula*, other mutant resources for reverse genetics are being developed in legumes. A TILLING platform is available for *L. japonicus* (Perry et al., 2003; Henikoff et al., 2004) and equivalent resources will soon be ready for pea, *M. truncatula*, and soybean (*Glycine max*; www.eugrainlegumes.org; www.soybeantilling.org). Also, a fast-neutron mutagenesis platform for reverse genetics screens will soon be available for *M. truncatula* (Li et al., 2001; www.eugrainlegumes.org). Obviously, mutations that will derive from these three approaches, *Tnt1*, fast neutron, and TILLING, have very different characteristics and that makes them useful for different purposes. Point mutations can be relatively costly to identify, but TILLING should be useful to provide a range of mutant alleles of different degrees of severity. Detecting mutations in a sequence when caused by insertions or deletions should be easier. These mutations tend to cause null alleles and those can be useful to understand the function of genes; however, they can cause lethal phenotypes. A clear advantage of *Tnt1* populations is that the cloning of genes identified through mutations in forward genetics screens is easier than in ethyl methanesulfonate or fast-neutron populations.

What seems clear is that a variety of complementary strategies for mutant isolation through reverse genetics will be efficiently working in model legumes very soon. This will greatly ease the dissection of genetic networks controlling traits of interest in these species and, in general, will allow rapid progress in the understanding of the biology of this family of plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Medicago truncatula (ecotype R108) was grown in the greenhouse at 22°C (day) and 18°C (night); 16-h photoperiods were maintained with supplementary lighting [400-W Phillips HDK/400 HPI (R) (N)]. Plants were grown in a mixture of soil:sand (3:1) and were irrigated with Hoagland no. 1 solution supplemented with oligoelements (Hewitt, 1966).

PCR-Based Screen of a *Tnt1* Mutant Population

The *M. truncatula* population used for the PCR-based screening of mutants was described in detail (d'Erfurth et al., 2003). Briefly, the genomic DNAs from 200 primary transformant plants, containing the *Tnt1* element, was extracted and pooled in 20 samples (10 plants/pool). PCR was performed with these DNA pools as templates, using degenerate oligonucleotides, annealing to the MADS-box conserved region (MAD2 and DAM1), and specific oligonucleotides (LTR6 and LTR31), annealing to the LTR borders of the *Tnt1* element. The degenerate oligonucleotides were designed by aligning available MADS-box sequences from The Institute of Genomic Research Medicago Gene Index.

Primer sequences were as follows: MAD1, 5′-ATGGGRAGRGGAAARATTGARATMAARAGGAT-3′; MAD2, 5′-ATGGGRAGRGGAAGAGTGSARTTGAARAGGAT-3′; MAD3, 5′-ATGGGNMGNGGNAARATHGA-3′; DAM1, 5′-ATCCTYTTKATYTCAATYTTTCCYCTYCCCAT-3′; DAM2, 5′-ATCCTYTTCAAYTSCACTCTTCCYCTYCCCAT-3′; LTR6, 5′-GCTACCAACCAAACCAAGTCAA-3′; and LTR31, 5′-CTCCTCTCGGGGTCGTGGTT-3′.

Isolation and Sequence Analysis of MtPIM cDNA

A cDNA library of *M. truncatula* (ecotype Jemalong A17) flowers and inflorescence apexes was generated (HybriZAP-2.1 XR Library; Stratagene) and screened at high stringency (65°C, 0.1 × SSC/0.1% SDS) with a 767-bp 3′

fragment of *PEAM4* cDNA (Berbel et al., 2001). Four independent clones were obtained, the longest one containing 1,152 bp with an open reading frame of 783 bp. The sequence of the Jemalong A17 *MtPIM* cDNA was identical to the *MtPIM* fragment amplified in the screening of the R108 *Tnt1* population. The comparison between nucleotide and protein sequences was performed with Alignn and Alignp software, respectively, provided on the Infobiogen Web site (http://www.infobiogen.fr/services/menuserv.html).

Genomic DNA Extraction and Southern-Blot Analysis

Plant genomic DNA was extracted from leaves as described (Dellaporta et al., 1983). Ten micrograms of DNA were digested with restriction enzymes and separated on 0.6% Tris-acetate EDTA 1 × agarose gels overnight at 1 V/cm. Southern-blot analysis was performed by standard methods. The probe was an *Eco*RI/*Xho*I restriction fragment, which includes the whole *MtPIM* cDNA.

Cosegregation Test

PCR reactions were performed using genomic DNA as a template 20 ng from each F2 individual plant (30 cycles; 58°C annealing temp). The presence of the *Tnt1* insertion in the *MtPIM* locus was tested by the amplification of a 301-bp band with the oligonucleotides Mtm42, 5′-AGGATAGAAAACAA-GATCAATCG-3′ (nucleotides 28–51 of the coding sequence of *MtPIM* cDNA) and LTR51, 5′-AAAGCTTCACCCTCTAAAGCCT-3′ (nucleotides 178–200 of the LTR of *Tnt1*), and the absence of the *Tnt1* insertion was tested by the amplification of a 156-bp band with the oligonucleotides Mtm42 and Mtm460, 5′-AGAATCAGTTGCATATTCAAAGAG-3′ (nucleotides 160–183 of the coding sequence of *MtPIM* cDNA).

Northern-Blot Analysis

Total RNA was isolated by phenol-chloroform extraction and precipitated with 3 M lithium chloride. RNA was electrophoresed in formaldehyde-agarose gels, transferred to Hybond N^+ membranes (Amersham Biosciences), and hybridized with ^{32}P under standard conditions. The probe was the 434-bp-long fragment of the C-terminal region of *MtPIM* cDNA amplified with the oligonucleotides AP1insF, 5′-ATAGCGGACTGAAGGCAAAG-3′ and MtAP1insR, 5′-GCATCCAAGATGGCAGGTAT-3′.

RNA in Situ Hybridization

RNA in situ hybridization with digoxigenin-labeled probes was performed on 8-μm longitudinal paraffin sections of *M. truncatula* inflorescences as described in Ferrándiz et al. (2000). RNA antisense and sense probes were generated with the SP6 and T7 polymerases, respectively, using as substrate the 434-bp 3′ region of the *MtPIM* cDNA, cloned into the pGemT-Easy vector (Promega). The hybridization signal was revealed by a purple precipitate when viewed under a light microscope. The specificity of the hybridization signal was tested either by probing wild-type inflorescences with the sense probe or by probing *mtpim* inflorescences with the antisense probe; no hybridization was observed in either case.

Light Microscopy and SEM

Light photographs of wild-type and *mtpim* mutant flowers were obtained using a dissection microscope (Leica MZ8). Specimens were freshly harvested and dissected using a forceps and scalpel. For SEM, samples were harvested, dehydrated, dried, and analyzed as described in Benlloch et al. (2002).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ139345.

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